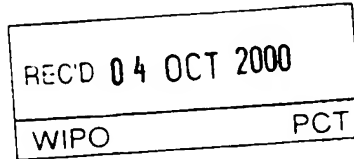




INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

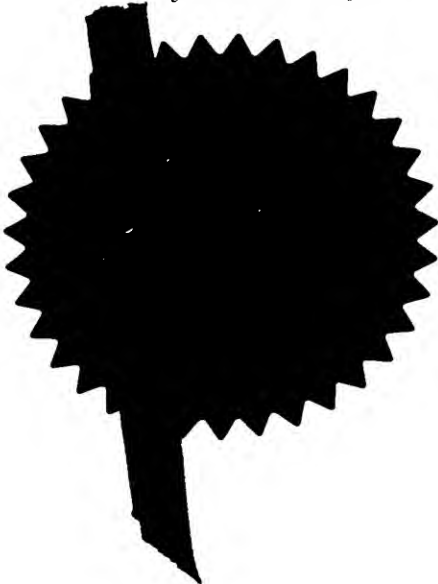


I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

Dated 20 September 2000

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



19 AUG 1977

Request for grant of a patent

See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form.

The Patent Office

Cardiff Road
Newport
Gwent NP23 5RH

Page 1

1. Your reference

NSM FIB 40878

2. Patent application number

(The Patent Office will fill in this part)

9919713.9

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Queen Mary and Westfield College
University of London
Mile End Road,
London
EC1A 4NS

Patents ADP number (if you know it)

0557326601

If the applicant is a corporate body, give the country/state of incorporation

United Kingdom

4. Title of the invention

New Medical Use of High Density Lipoprotein

5. Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent

Reddie & Grose
16 Theobalds Road
LONDON
WC1X 8PL

Patents ADP number (if you know it)

91001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application
(If you know it)

Date of filing
(day/month/year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day/month/year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

- a) any applicant named in part 3 is not an inventor, or
 - b) there is an inventor who is not named as an applicant, or
 - c) any named applicant is a corporate body.
- See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document.

Continuation sheets of this form

Description	12
Claim(s)	0
Abstract	0
Drawing(s)	04 + 4 <i>14</i>

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent (<i>Patents Form 7/77</i>)	0
Request for preliminary examination and search (<i>Patents Form 9/77</i>)	0
Request for substantive examination (<i>Patents Form 10/77</i>)	0
Any other documents (<i>please specify</i>)	0

11. I/We request the grant of a patent on the basis of this application.

Signature	Date
<i>Reddie & Grose</i>	19 August 1999

12. Name and daytime telephone number of person to contact in the United Kingdom
- P I BATES - Reddie & Grose
0171-242 0901

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or such direction has been revoked.

Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

NEW MEDICAL USE OF HIGH DENSITY LIPOPROTEIN

The present invention relates to the manufacture of medicaments for protecting against organ damage following ischaemia-reperfusion injury, using high-density lipoproteins HDLs and derivatives thereof. In particular, it relates to manufacture of medicaments for treatment and prevention of end-stage organ failure in haemorrhagic shock, and for treatment and prevention of tissue injury following myocardial infarction.

10 Many victims of sudden physical injury (for example, traffic accident victims) die because of end-stage organ failure. In patients with this condition, biochemical and biological changes (such as haemodynamic changes and microthrombus formation) occur in the blood and organs (such as liver and
15 kidneys) due to shock and blood loss; this is a different action to "endotoxin" shock which arises due to bacterial infection. If end-stage organ failure is not halted or prevented, it will lead to permanent organ damage and death of the patient. There is a need for a pharmaceutical agent
20 which can be administered as soon as possible after the physical injury, preferably at the site of the accident in order to prevent end stage organ failure, and which can also be used subsequently while transporting the victim from the accident site to casualty/hospital, and while the physical
25 wounds are being treated.

High-density lipoproteins (HDLs) form a range of lipoprotein particles found in normal serum. Mature HDL particles are present in the form of a globular structure containing proteins and lipids. Within the outer layer of these
30 particles are the more polar lipids, phospholipids and free

cholesterol, all having charged groups pointing outwards towards the aqueous environment. The more hydrophobic lipids, such as esterified cholesterol and triglycerides, reside in the core of the particle. Newly formed, or nascent, HDL particles lack the lipid core and are discoidal in shape. Protein components are embedded in the outer layer. The main protein component is apolipoprotein A-I (apo A-I), with smaller amounts of apo A-II, apo A-IV, apo CIII, apo D, apo E and apo J. Various other proteins reside on the HDL particle, such as lecithin-cholesterol acetyltransferase, PAF acetylhydrolase and paraoxonase.

The binding of activated leukocytes to the endothelium is the earliest observable cellular event in a number of acute and chronic inflammatory diseases. This binding is mediated by the expression of adhesion molecules on the surface of the endothelial cells which bind to corresponding molecules of similar function on leukocytes. Recently we have shown that pre-treatment of endothelial cells, *in vitro*, with HDL was able to inhibit the cytokine-induced expression of these adhesion molecules (Cockerill GW, Rye K-A, Gamble JR, Vadas MA, Barter PJ. *Arterioscler Thromb. Vasc. Biol.* 1995, 15: 1987-1994 1995, Cockerill GW Reed S. *Int.Rev.Cytol: A survey of cell biology* 1999). In addition, we have recently shown that HDL can inhibit cytokine-induced adhesion molecule expression in an acute inflammatory model in the pig (Cockerill et al., submitted 1999). The antiinflammatory effects of HDL have thus been demonstrated in these models where cells/animals are pre-treated with lipoprotein.

End-stage organ failure following haemorrhagic shock results from the adhesion of polymorphonuclear leukocytes (PMNs) to the endothelium following their activation caused by

- 7 -

ischaemia and reperfusion injury. We have now found that administration of HDL or derivatives thereof prevents end-stage organ failure following ischaemia and reperfusion injury.

According to the present invention high density lipoprotein and or a derivative thereof is used in the manufacture of a medicament for the prevention or treatment of organ dysfunction following ischaemia and reperfusion injury.

Preferably, the medicament is for the treatment of end-stage organ injury or failure.

We have shown that, following ischaemia perfusion injury, HDL is able to perturb the damaging effects when given after the initial haemorrhagic shock has occurred. Our work suggests that at physiological levels (both *in vitro* and *in vivo*), native HDL particles are active in inhibiting the expression of adhesion proteins on endothelial cells. Prevention of expression of adhesion proteins on endothelial cells prevents binding of PMNs to the endothelium; thus administration of HDL prevents end-stage organ failure.

The high density lipoprotein may be the component of HDL that inhibits adhesion to the endothelial cells and subsequent activation of leukocytes or a derivative, molecule, homologue, or mimic thereof.

The inhibiting effect is not only present in venous endothelial cells but also on arterial endothelial cells and is independent of the nature of the lipid present in the HDL particles. Two effector molecules mediate the inhibitory effect namely apolipoprotein A-I and apo A-I and

apolipoprotein A-II (apo A-II) (Brouillette C.G. and Anatharamaiah G.M. Biochem.Biophys. Acta. 1256: 103-129. 1995; Massey J.B., Pownall H.J. Biochem.Biophys Acta. 999 : 111-120. 1988); these two molecules have different efficacy of inhibition.

Preferably, the high density lipoprotein or derivative thereof is a peptide or protein derivative of the sequence of apo A-I or apo A-II, or a peptide or protein derivative functionally homologous to the active portions of apo A-I or apo A-II.

Preferably, the high density lipoprotein is reconstituted HDL.

The medicament may be administered to a patient in any conventional manner. Preferably the medicament is administered intravenously. Preferably, the medicament is administered using saline as a vehicle.

Preferably the medicament is provided in a portable dispenser, for example, for use at the site of an accident.

According to the invention in another aspect there is provided a method of treatment of organ dysfunction following ischaemia and/or reperfusion injury in a human patient which comprises the step of administering to a patient reconstituted high density lipoprotein and/or a derivative thereof in pharmaceutically acceptable form.

The present invention will now be illustrated with reference to the attached drawings in which :

FIGURE 1 shows alterations in mean arterial blood pressure (MAP) in rats subjected to the surgical procedure without causing a haemorrhage and treated with vehicle for HDL (SHAM, open circle, saline, 3mg/kg i.v. bolus; n=9) or with HDL (SHAM + HDL black circle, 80mg/kg i.v. bolus injection, n=9) or after haemorrhage for 1.5 h and upon resuscitation with the shed blood, control rats were treated with the vehicle (HS open squares, saline 3ml/kg i.v. bolus; n=10) or HDL (HS + HDL filled squares, 80mg/kg i.v. bolus injection, n=9);

FIGURE 1A shows a Table of heart rate in beats per minute (bpm) in all experimental groups studied before the haemorrhage -1.5 h and 1, 2, 3 and 4 h after resuscitation. Group 1: Rats were subjected to the surgical procedure without causing a haemorrhage and treated with a vehicle for HDL (saline, 3mg/kg i.v. bolus; n=9); Group 2: Rats were subjected to the same surgical procedure as Group 1 but treated with HDL (80mg/kg i.v. bolus injection; n=9); Group 3: Rats were subjected to a haemorrhage for 1.5 h and upon resuscitation with the shed blood, control rats were treated with the vehicle (saline 1ml/kg i.v. bolus followed by an infusion of 1.5 ml/kg/h i.v., n=10); Group 4: rats were subjected to the same procedure as group 3 but treated with HDL (80mg/kg i.v. bolus injection; n=9).

FIGURE 2 shows plasma levels of (A) urea, (B) creatine, (C) AST, (D) ALT, (E) creatinine kinase (CK) and (F) lipase in rats subjected to the surgical procedure and experiment 2 described below;

FIGURE 3 shows a graph of mean fluorescence intensity (dependent on inhibition of E-selectin), as described below;

FIGURE 4(a) shows a graph of mean fluorescence intensity of HUVEC (veinous EC) against concentration of lipoproteins apo A-I and apo A-II for experiment 3, below; and

FIGURE 4(b) shows a graph of mean fluorescence intensity of HuAEC (arterial EC) against concentration of lipoprotein apo A-I and apo A-II for experiment 3, below.

As a demonstration of an embodiment of the invention, Experiment 1 describes the effects of human high-density lipoprotein (HDL) on the circulatory failure and multiple organ dysfunction injury (MODS) such as renal dysfunction and liver dysfunction caused by severe haemorrhage and resuscitation in the anaesthetised rat. It should be noted that this is a model of end stage organ failure generated by ischaemia and reperfusion injury, and is not known to be a result of endotoxin release.

All experiments described herein were performed in adherence to the National Institute of Health guidelines on the use of experimental animals and in adherence to *Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London.

Experiment 1

The study was carried out on 26 Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 250mg - 320g receiving a standard diet and water *ad libitum*. All animals were anaesthetised with thiopentone (120mg/kg i.p.) and anaesthesia was maintained

by supplementary injections of thiopentone as required. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right femoral artery was catheterised and connected to a pressure transducer (Senco-Nor 840, Senco-Nor, Horten, Norway) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR). These were displayed on a data acquisition system (MacLab 8e, ADI Instruments, Hastings, UK) installed on an Apple Macintosh computer. The right carotid artery was cannulated to bleed the animals (see hereafter). The jugular vein was cannulated for the administration of drugs. The bladder was also cannulated to facilitate urine flow and to prevent the possibility of development of post-renal failure. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 15 mins. Then, blood was withdrawn from the catheter placed in the carotid artery in order to achieve a fall in MAP to 50mmHg within 10 mins. Thereafter, MAP was maintained at 50mmHg for a total period of 90 mins by either withdrawal (during the compensation period) or re-injection of blood. It should be noted that in these experiments, the amount of shed blood re-injected during the 90 min period of haemorrhage did not exceed 10% of the total amount of the blood withdrawn. The amount of blood withdrawn for rats subjected to haemorrhage and treated with vehicle (control group) was 7.0 ± 0.4 ml (SD); the amount of blood withdrawn from rats subjected to haemorrhage and treated with HDL (treatment group) was 7.0 ± 0.3 ml ($p > 0.05$). At 90 min after initiation of haemorrhage, the shed blood and an equivalent volume of Ringer lactic solution was re-injected into the animal.

The results are shown in Figures 1, 1A (Table 1) and 2.

FIGURE 2 shows plasma levels of (A) urea, (B) creatinine, (C) AST, (D) ALT, (E) creatinine kinase (CK) and (F) lipase in rats subject to (i) the surgical procedure without causing a haemorrhage and treated with vehicle for HDL (sham+saline, saline, 3ml/kg i.v. bolus i.v.; n=9) or with HDL (sham +HDL; 80mg/kg i.v. bolus injection, n=9, or (ii) haemorrhage for 1.5.h and upon resuscitation with the shed blood, control rats were treated with the vehicle (hs (+ saline), saline 3ml/kg i.v. bolus n=9) or HDL (hs + HDL, 80mg/kg i.v. bolus injection, n=9). Haemorrhage and resuscitation resulted in significant increases in the serum levels of urea and creatinine (n=9), as demonstrated by the increase in urea and creatine concentration between "sham" and hs (control). This renal dysfunction was attenuated by the administration (5 mins prior to resuscitation) of HDL (80mg/kg. i.v., n=9; p,0.05; ANOVA followed by Bonferoni's test for multiple comparisons), as demonstrated by the concentration of urea and creatinine for "hs + HDL". Similarly, HDL attenuated the liver injury (as monitored by a rise in serum AST and ALT) - (C) and (D) - and the pancreatic injury (as measured by a rise in serum lipase - (F)) caused by haemorrhage and resuscitation. In contrast, HDL did not affect the delayed circulatory failure associated with haemorrhage and resuscitation (see Fig 1 and Fig.1A (Table 1)). Administration of HDL to rats, which were not subjected to haemorrhage, did not result in the alterations in the serum levels of urea, creatinine, AST, ALT or lipase (n=4) and, hence, was not toxic and the dose used.

In conclusion, administration of HDL attenuates the renal, liver and pancreatic dysfunction associated with ischaemia and reperfusion injury following haemorrhagic shock.

Experiment 2

This experiment demonstrates which components of the effective therapeutic agent HDL are responsible for protection against ischaemia-reperfusion injury; in this experiment, the ability of native HDL to inhibit cytokine-induced adhesion molecule expression on endothelial cells is compared with the ability of lipid-free apo A-I protein or protein-free lipid vesicles.

Cell culture : Human umbilical vein-derived endothelial cells (HUVEC) and human umbilical-derived artery endothelial cells (HUAEC) (Cockerill G.W, Meyer G, Noack L.Vadas MA, Gamble J.R. Lab.Invest.71 : 497-509.1994) were grown on gelatin-coated tissue culture flasks (Costar, High Wycombe, Bucks, UK) in medium 199 with Earle's salts (Gibco, Paisley, Scotland) supplemented with 20% fetal calf serum (FCS) (Gibco, Australia), 20mM HEPES, 2mM glutamine, 1mM sodium pyruvate, non-essential amino acids, penicillin and streptomycin, 50µg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 50µg/ml heparin (normal growth medium).

Flow cytometry : Cells were plated at 1×10^5 cells/30 mm well and incubated overnight at 37°C in 5% CO₂. Confluent monolayer cultures were then incubated (at concentrations indicated) for 19 hours with either, phosphate buffered saline (PBS) (vehicle control), native HDL, free apo A-I, phospholipid vesicles or discoidal HDL prepared with only apo A-I or apo A-II. Following these treatments the cells were washed gently in complete medium and TNFα (Miles Scientific) was added at 10ng/ml. Cells were then stained at 4 hours post stimulation in the following manner. Cells were washed in serum free medium and 200µl anti-E-selectin

(1,2B6) was added for 1 hour at 37°C. Cells were then washed in phosphate buffered saline (PBS) containing 5% newborn calf serum, 0.02% sodium azide, and 200µl of fluorescein isothiocyanate-conjugated secondary antibody added for 1 hour at 37°C. Cells were then washed three times in PBS and trypsinised, then centrifuged to form a pellet. The pellet was then resuspended in 2.5% formaldehyde in PBS containing 2% glucose and 0.02% azide and analyzed in a Coulter Epics Profile II flow cytometer.

Figure 3 shows that neither free apo A-I nor unilamellar vesicles (SUV) were able to inhibit TNFα-induced expression of E-selectin. This suggests that Apo A-I, the most abundant apolipoprotein in HDL, must be in a lipid particle in order to mediate inhibition of cytokine-induced adhesion molecule expression in endothelial cells. Both umbilical-derived venous (HUVEC) and arterial (HUAEC) endothelial cells were able to support the dose-dependent inhibition of cytokine-induced E-selectin expression by HDL (as shown by the decrease in intensity with increase of apo AI HDL from 0.25 to 1.0mg/ml).

The therapeutic action of HDL is afforded by the apolipoprotein presented in a lipid particle, and cannot be mimicked by the whole protein alone, or lipid alone.

Experiment 3

To determine the efficacy of reconstituted discoidal HDLs particles containing either of the most abundant apolipoproteins (apo A-I or apo A-II), a comparison of the ability of these particles to inhibit cytokine-induced

adhesion molecule expression in HUVEC and HUAEC was carried out.

Cell culture : Human umbilical vein-derived endothelial cells HUVEC and human umbilical-derived artery endothelial cells HUAEC (Cockerill et al., 19994) were grown on gelatin-coated tissue culture flasks (Costar, High Wycombe, Bucks, UK) in medium 199 with Earle's salts (Gibco, Paisley, Scotland), supplemented with 20% foetal calf serum (FCS (Gibco, Australia), 20 mM HEPES, 2mM glutamine, 1mM sodium pyruvate, nonessential amino acids, penicillin and streptomycin, 50µg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 50µg/ml heparin (normal growth medium).

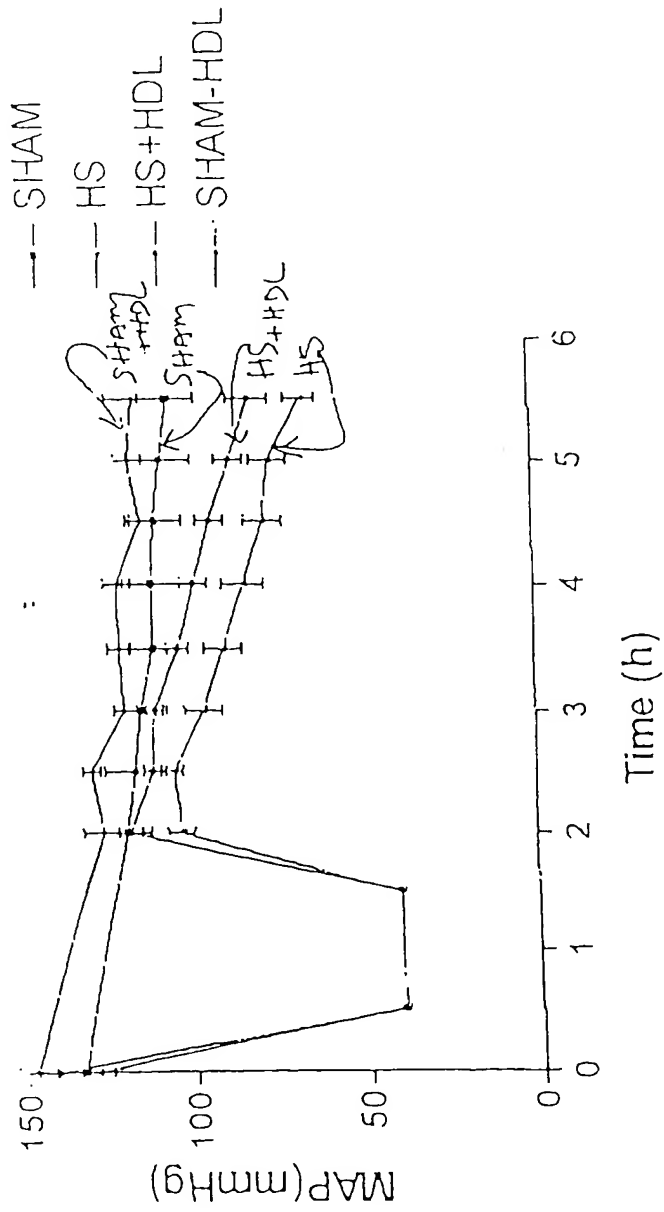
Flow cytometry : Cells were plated at 1×10^5 cells/30 mm well and incubated overnight at 37°C in 5% CO₂. Confluent monolayer cultures were then incubated (at concentrations indicated) for 19 hours with either reconstituted discoidal HDL prepared with only apo A-I or apo A-II. Following these treatments the cells were washed gently in complete medium and TNFα (Miles Scientific) was added at 10ng/ml. Cells were then stained at 4 hours post stimulation in the following manner. Cells were washed in serum free medium and 200µl anti-E-selectin (1.2B6) was added for 1 hour at 37°C. Cells were then washed in phosphate buffered saline (PBS) containing 5% newborn calf serum, 0.02% sodium azide, and 200µl of fluorescein isothiocyanate-conjugated secondary antibody added for 1 hour at 37°C. Cells were then washed three time in PBS and trypsinised. The pellet was then resuspended in 2.5% formaldehyde in PBS containing 2% glucose and 0.02% azide and analyzed in a Coulter Epics Profile II flow cytometer.

Preparation of Reconstituted HDL Particles : Discoidal reconstituted A-I HDLs were prepared by the cholate dialysis method from egg yolk phosphatidylcholine, unesterified cholesterol, and apo A-I/apo A-II (Matz CE, Jonas A. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersion. *J.Biol.Chem.*1982; 257; 4535-4540). Egg yolk phosphatidylcholine, unesterified cholesterol and sodium cholate were obtained from Sigma and used without further purification. Particle size was measured by nondenaturing gradient gel electrophoresis, and concentration of apo A-I and apo A-II was measured immunoturbidimetrically.

Results : Discoidal reconstituted HDL particles containing either apo A-I (open squares) or apo A-II (closed squares), as the sole protein, were able to inhibit TNF α -induced expression of both arterial and venous endothelial cells VCAM-1. Figure 4 shows reconstituted HDL containing apo A-I, as the sole proteins, having a t_{1/2} max of approximately 3 μ Molar, whilst reconstituted HDL containing apo A-II as the sole protein has a give five-fold greater t_{1/2} max of 15 μ Molar.

Conclusion : The therapeutic action of HDL can be mimicked using either apo A-I or apo A-II in reconstituted lipoprotein particle.

Figure 1

Figure 1A
(Table 1)

Group	-1.5 h	1 h	2 h	3 h	4 h
1. sham + saline	344±12	350±9	364±10	360±14	380±17
2. sham + HDL	380±14	397±12	399±7	396±9	384±16
3. HS + saline	368±15	397±10	380±18	365±16	345±23
4. HS + HDL	352±9	362±13	380±13	381±9	382±11

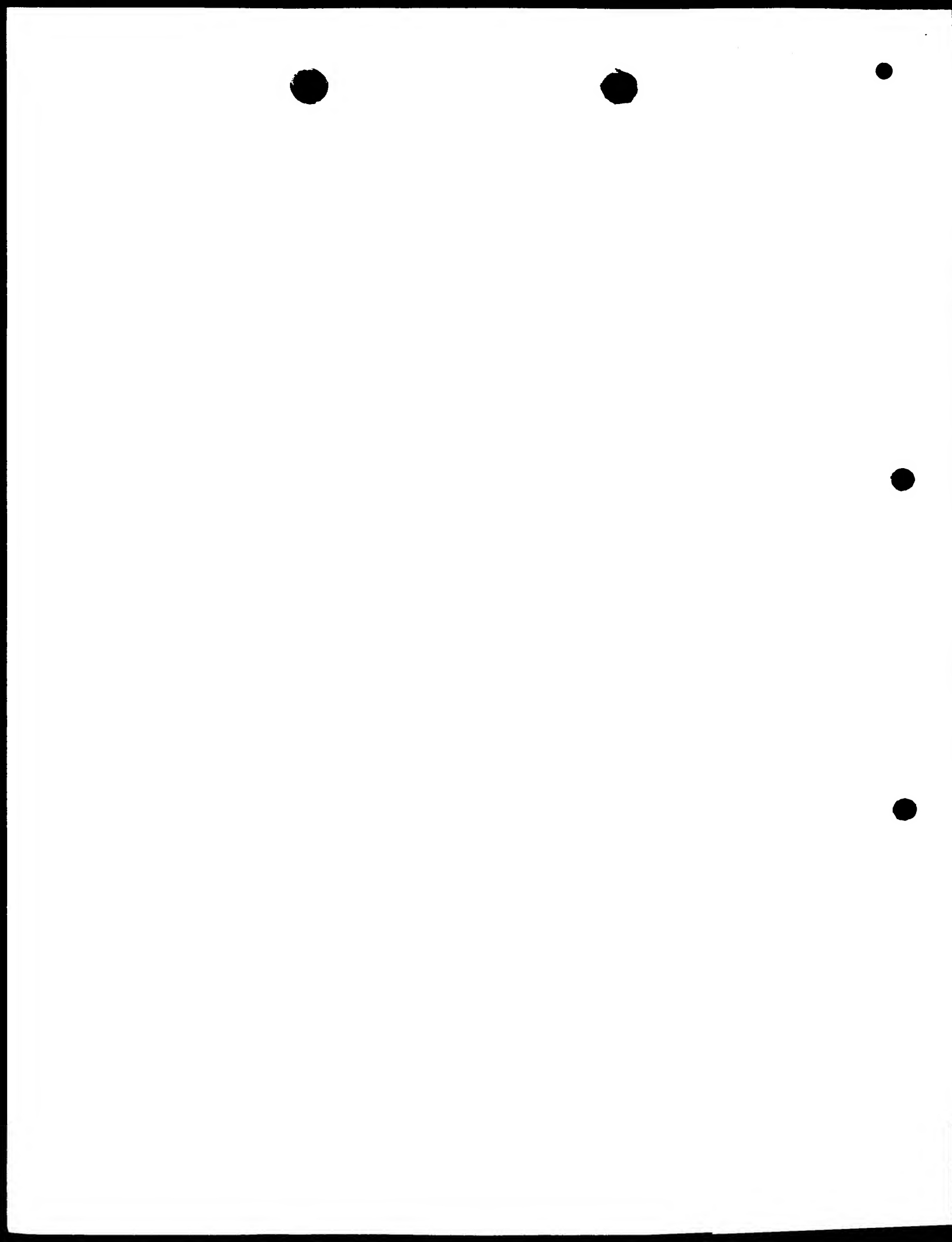


Figure 2A

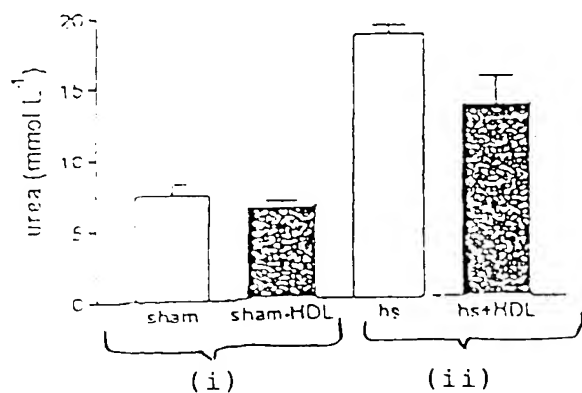


Figure 2B

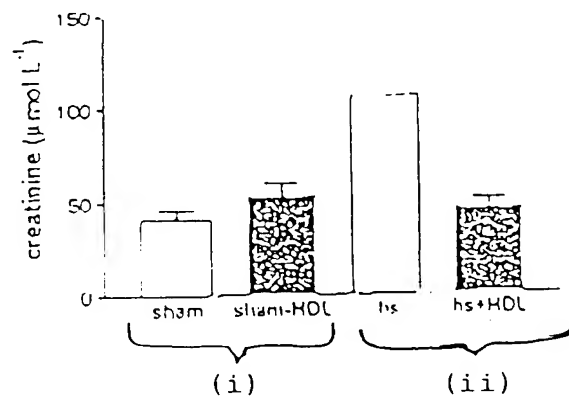


Figure 2C

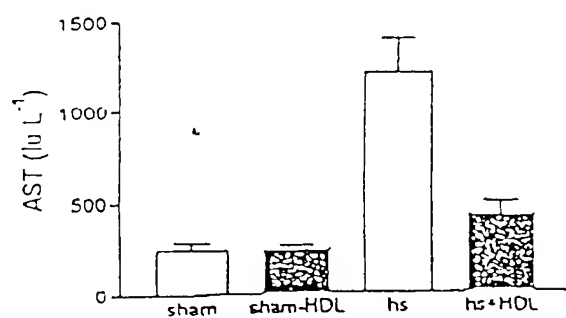


Figure 2D

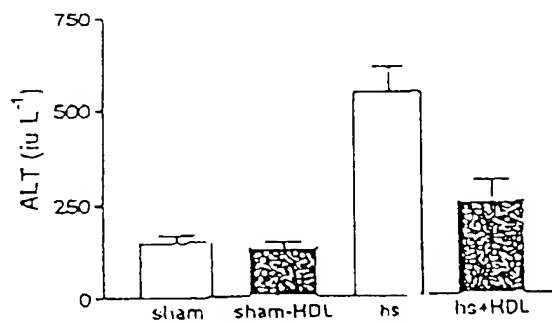


Figure 2E

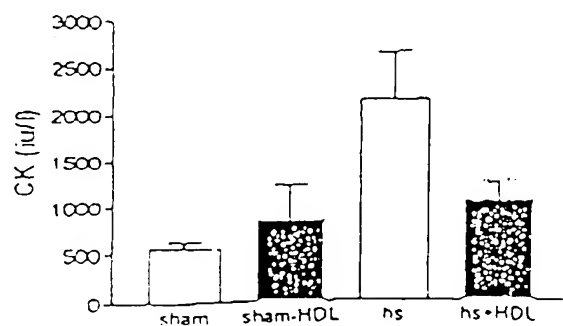


Figure 2F

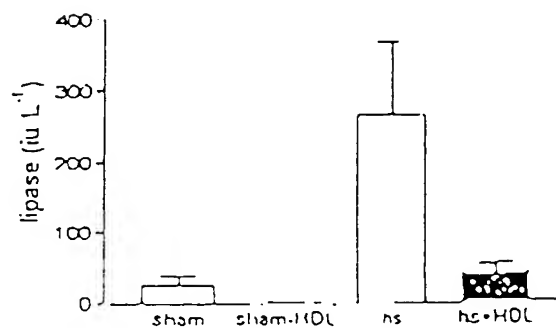




Figure 3

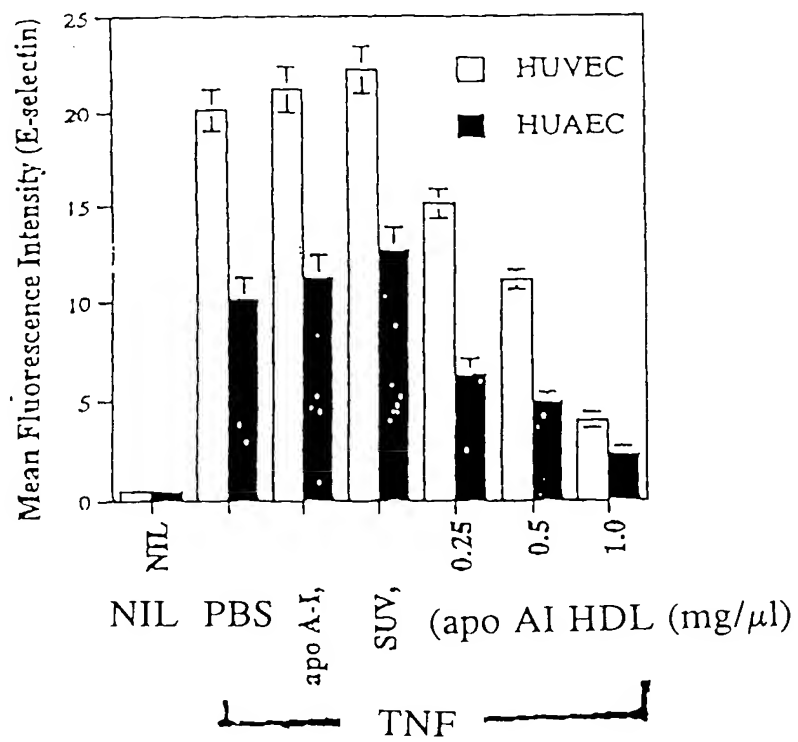




Figure 4A

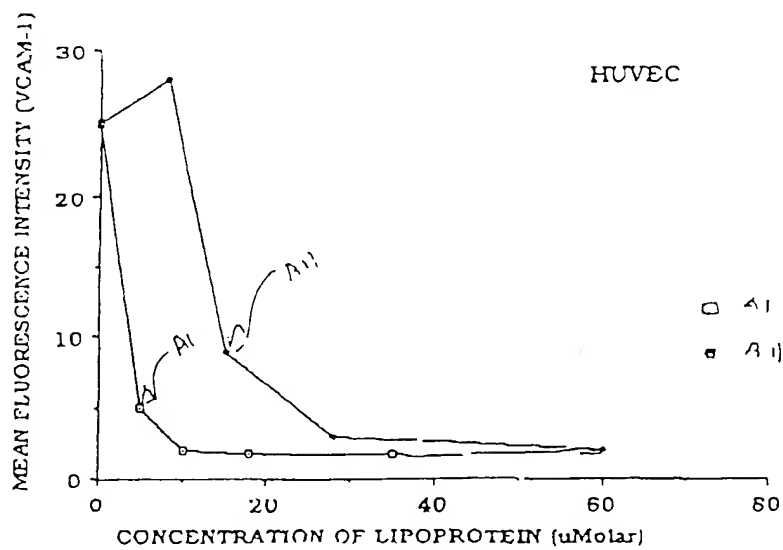
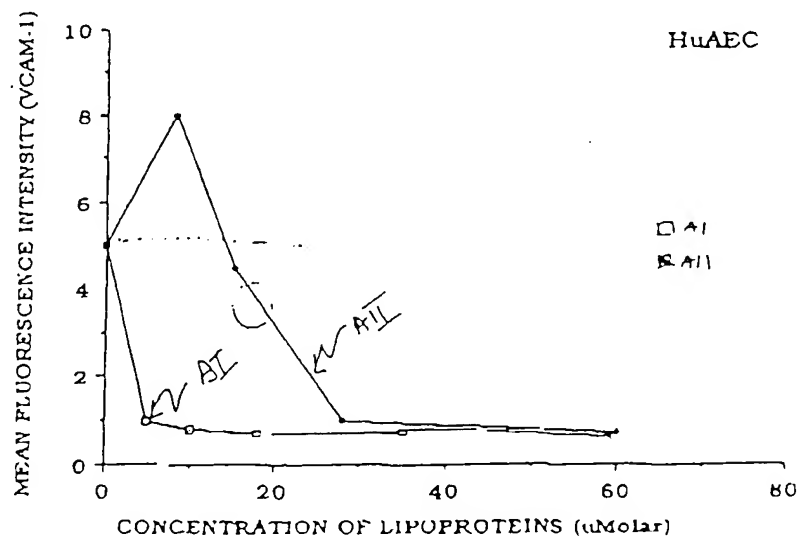


Figure 4B





11-11-11

11-11-11

